Cell Wall Cross-linking in Grasses

The Importance of Understanding Plant Chemistry and Biochemistry

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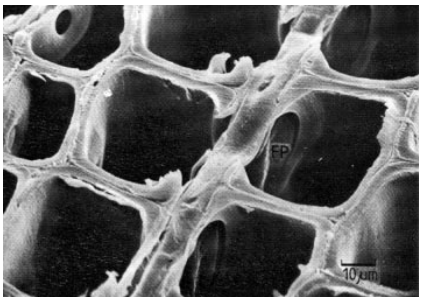

Content

Introduction

Plant cell walls form a large part of the diet of forage-fed animals. The US Dairy Forage Research Center is committed to answering questions that will result in increased economical and efficient utilization of forages. Among these are efforts that expand our understanding of the chemistry, biochemistry, growth and development of forages, particularly as these areas relate to forage utilization by ruminant animals. Improving forages for dairy production requires selection of plants with increased cell wall digestibility. The avenues which we take to reach this goal are varied and largely uncharted. The area has become particularly exciting and we have been able to make significant contributions over the past few years. Our efforts focus on identification of molecular mechanisms that ultimately limit cell wall digestibility. Such information clearly identifies structural limitations to wall degradation and in most cases identifies the biochemical process responsible. This in turn identifies the appropriate avenues that seem the most beneficial to pursue. This Chapter illustrates the types of information being sought; how this is utilized in others' research is seen in some of the other Chapters. We believe these collaborative studies provide a sound basis for the application of emerging technologies to improve plants for targeted uses.

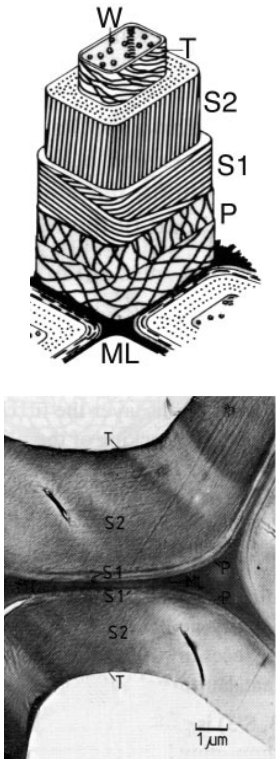

Figure 1. Top: Model of the cell wall structure. Bottom: Transmission electron micrograph of ultrathin section of cell walls showing the various wall layers: ML = middle lamella, M = compound middle lamella, P = primary wall, S1 = secondary wall 1, S2 = secondary wall 2, T = tertiary wall.

Lignin may be produced as a property-oriented polymer. Exact structure may not be that important.

Plant Cell Walls

The plant cell wall is a major component of terrestrial plants, providing structural strength in our gravitational environment and other important functions. Ruminant animals, with the aid of rumen microorganisms, are capable of digesting and degrading cell wall polysaccharides, a feat at which humans (and other non-ruminants for that matter) fare very poorly. Thus the cell wall is a significant source of nutrition for the animal. But what more do we need to know about the cell wall? It contains carbohydrate polymers (polysaccharides) including cellulose, hemicelluloses, and pectins, as well as a rather flamboyant, if at times unpopular, non-carbohydrate polymer, lignin. What more needs to be known? And why should we care about lignin since it is simply indigestible anyway? Well — it turns out that there is tremendous interest in lignin again, particularly following the human race's recently acquired ability to mess directly with plant genes.

Lignin

Lignin has always been considered an enigma in the natural world (Harkin 1973). It is a polymer with no defined structure, no regularly repeating sequences of any length, and ill-defined size. However, we (in the US Dairy Forage Center's Cell Wall Group) are beginning to believe that it is only one of a number of polymers that plants create with little regard to exact order but to produce polymers with certain basic properties. As an analogy, consider that we may have had plans for and wished to build a garden shed from Maple. If Maple became unavailable or was just too expensive for us, we could use a cheaper soft pine quite satisfactorily. We might have to use more of it, perhaps with greater thicknesses, and we may choose to brace it more extensively, but there would be no problem building essentially the same type of shed. If for some reason, wood became completely unavailable, we could even make our shed quite satisfactorily from planks of plastic.

It is becoming clear to us (although other groups are still firmly entrenched in more traditional ideas) that the plant system is similarly adaptable with respect to lignin. For example, the major lignin building block is a simple natural chemical called coniferyl alcohol, Fig. 2. Through genetic engineering, by using anti-sense genes to the CAD enzyme, the production of coniferyl alcohol can be almost completely turned off. This, researchers surmised, would prevent a plant from growing properly. Just down-regulating that gene a little might therefore lower the amount of lignin in the plant (and consequently make it more digestible etc.). Imagine their surprise when the plants deprived of their ability to make coniferyl alcohol grew perfectly well and seemed to produce lignin. In an anthropomorphic way, the plant simply said, "Yikes, what's going on; I can't seem to make coniferyl alcohol. Oh well, I seem to be able to make the precursor, coniferaldehyde just fine; I'll just make lignin out of that!" And it does. The lignin has some different properties, so the plant has to make a few other adjustments, but perfectly viable plants are produced. In the same vein, another gene has been targeted. That is the one that affects the final step in producing the next major lignin building block, sinapyl alcohol, Fig. 2. It has not been possible to down-regulate this OMT enzyme to the same high extent, but again, the plant doesn't really care — it just says, "Oops, I'm feeling a bit out of sorts and just can't seem to get through this pathway all the way; I can't seem to make sinapyl alcohol fast enough. Oh well, I'll just ship out the unfinished product (5-hydroxyconiferyl alcohol, Fig. 2) and hope the wall synthesis crew can use that. Maybe the boss won't notice." Again, the plant makes a lignin incorporating this compound. This may be a bit of a disappointment to the gene jockeys but, thanks to the basic work that had been done on lignin formation mechanisms, it is not at all surprising to the lignin chemist. As long as we agree that the plant just needs a building material with appropriate properties, it is not overly critical what goes into it.

In fact, plants have already explored some of these options. Sederoff's group,

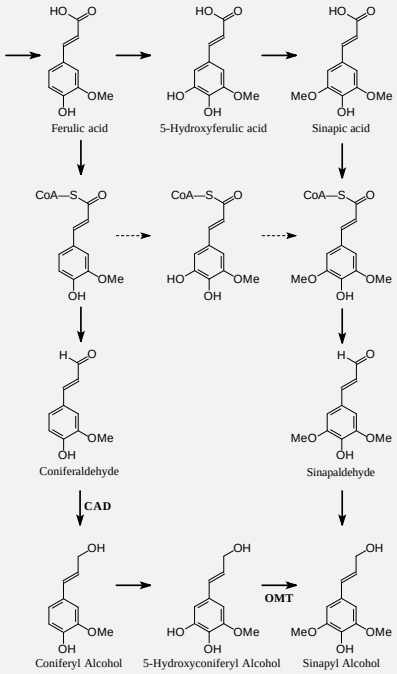

Figure 2. Partial pathway to the lignin monomers coniferyl and sinapyl alcohols. If the CAD enzyme is suppressed, coniferaldehyde cannot be converted to coniferyl alcohol, so the plant just makes its lignin using coniferaldehyde. Similarly, if the OMT enzyme is suppressed, 5-hydroxyconiferyl alcohol cannot be completely converted to sinapyl alcohol, so the plant incorporates the 5-hydroxyconiferyl alcohol into its lignin.

Cross-linking has a major impact on properties.

for example, has identified rigorously growing natural pine mutants that are almost entirely lacking in the CAD gene (Fig. 2). We have begun a study looking at the natural and mutant lignins to determine just how these pines successfully produce a lignin that is clearly functioning well but cannot contain what is considered to be the prime building block for lignin. In view of the coloration of these woods, we suspect that the lignins are made from coniferaldehyde, Fig. 2.

We have been interested in lignin less for itself than for the way it can protect carbohydrates from digestion. For example, even if cell wall materials (or collected fecal material that has already been digested once by the cow) are digested for an essentially infinite time in rumen conditions, the material that remains undigested is still two-thirds carbohydrate (Mertens and Hatfield 1992). That is, two-thirds of it is still potentially digestible. And, if you analyze its composition, it is strikingly similar to the original plant material. So why can't this polysaccharide be fully degraded? It could be physical effects (e.g., lignin encrustation); we certainly don't want to forget the importance of physical factors, but that is a mind-numbingly boring possibility to study! The alternative is that lignin may somehow be chemically bonded to the carbohydrate component; that is, the two fractions (polysaccharides and lignin) may be cross-linked.

Cell Wall Cross-linking

So, how much cross-linking is required to have an effect? A common-day illustration is a useful polymer called polyisoprene, better known as rubber. As it comes out of trees in Malaysia, for example, the milky juice (latex) is easily polymerized by drying in sunlight to form soft flexible sheets. These are very elastic, but tear easily, succumb quickly to abrasion, and swell up immensely, almost dissolving, in gasoline. Apply a few sulfur-mediated cross-links in the vulcanizing process, however, and you have a material that you will complain about when it can only endure 50,000 miles in contact with the road (your mileage may vary!). The point is that a few cross-links can effect major property changes.

Fig. 3 shows how a cow could easily push over a square structure. Apply a few cross-braces, however, and it is no longer such a pushover. Plant cell walls, as it turns out, use a remarkably similar approach, Fig. 4. They strategically place (ester-bonded) ferulates on some fractions of their polysaccharides and then couple these together to tie two polysaccharide chains together. In the absence of the chemical cross-braces, digesting the wall is a pushover to the cow. But the cross-links make substantial chunks of the polysaccharide unavailable to digesting enzymes and inhibit access to other chunks.

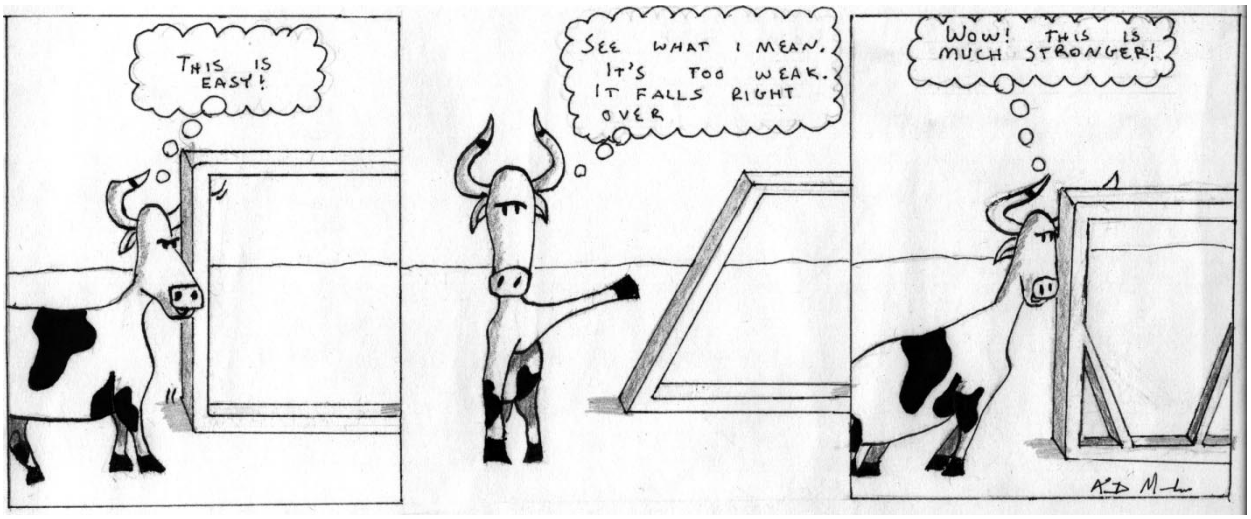

Figure 3. A square structure is a pushover for a cow. If that structure is braced ('cross-linked') it becomes much stronger.
Cartoon by nephew Andy Muenchow, 13.

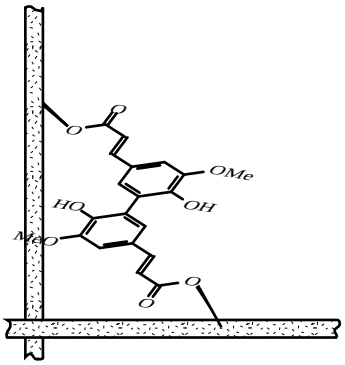

Figure 4. Cross-linking of polysaccharide chains in the cell wall by diferulates.

"Cross-linking by diferulates has been underestimated by factors of up to 20!"

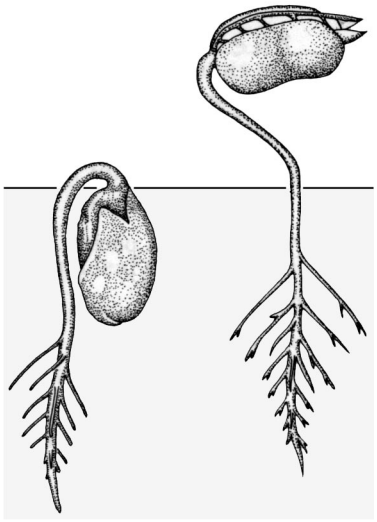

Figure 6. When a growing hypocotyl reaches light, it stops extending and begins leaf development. Cross-linking the hypocotyl's polysaccharides using diferulates is how the plant effects this abrupt halt to elongation.

Polysaccharide-Polysaccharide Cross-linking by Ferulates

What is the mechanism for this cross-linking by ferulate? And why might it be important to know? For almost 20 years now, scientists have known about a diferulate, a dimer made by chemically joining two ferulates more correctly called 5,5-diferulate (the numbers describe how ferulate units are attached), and it has recently been shown to cross-link polysaccharide chains. This 5,5-diferulate has been quantitated for many years and was known to be at rather low levels, so low in fact that we chose initially to ignore it in favor of other aspects we deemed to be more important. Our contribution came from trying to understand the mechanisms of cross-linking by ferulates.

Although it is well known that lignin is formed when plant enzymes modify coniferyl and sinapyl alcohols to cause them to combine, for some reason the structurally rather similar ferulate was not thought of in these terms. Yet, if 5,5-diferulate is formed from ferulate, it is hard to envision any mechanism other than radical coupling. The problem for us was that we could not understand why such radical coupling would produce only the 5,5-coupled compound. And, when we took model ferulates which mimic those in the cell wall but without the long polysaccharide chain attached, they coupled in a variety of other ways, producing so-called 8,5-, 8,8- and 8-O-4-coupled diferulates, but we failed to produce any significant amounts of the 5,5-diferulate. How was it that the plant was able to produce just this compound? Was it because the coupling was under some sort of tight enzymatic control?

It turns out that the plant does actually produce the whole range of diferulates, Fig. 5, and the 5,5-diferulate is not even major (Ralph, Quideau et al. 1994). The other compounds had simply been overlooked for the past 20 years. By synthesizing each of these diferulates and providing methods for their quantitation, we have shown that diferulates are far more significant than ever realized. They had been underestimated by factors of up to 20 and, rather than comprising just a few percent of the total ferulates in the wall, actually account for up to 70%. And all these revelations arose out of simply considering mechanisms.

But what good comes from knowing this? Some of these issues and rationales are more fully considered in following papers. The major point here is that ferulate has been identified as having a major role in cross-linking wall polysaccharides to each other. We have been able to show (peek ahead to Figure 10_{left}) that cross-linking polysaccharides by the formation of diferulates has a significant role in reducing both the rate and extent of polysaccharide degradability (Grabber, Hatfield et al. 1995). And an even larger role for ferulates and diferulates is still to come (below). Since the 'discovery' of these other diferulates, groups around the world are finding even more intriguing roles for this cross-linking. As examples, the cessation of hypocotyl elongation

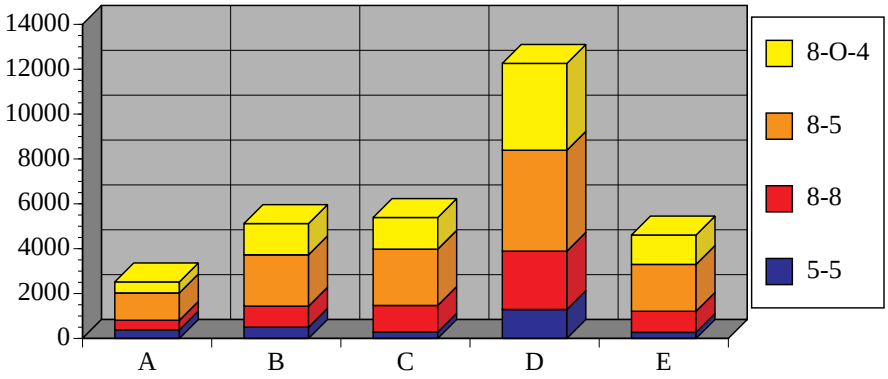

Figure 5. Composition of diferulates for various plant cell wall samples: (A) suspension-cultured corn, (B) cocksfoot parenchyma, (C) cocksfoot sclerenchyma, (D) switchgrass parenchyma, (E) switchgrass sclerenchyma.

that occurs when sprouting seedlings encounter light (Fig. 6) has been shown to be orchestrated by ferulate dimerization (Sánchez, Peña et al. 1996), and a British group claims that the crunchiness of water chestnuts in Chinese cuisine (even after substantial cooking) is due to diferulates (Parr, Waldron et al. 1996; Waldron 1996). How is that for appetizing!

Do Ferulates Cross-couple with Lignin?

Ferulate turns out to be even more important in grass walls. Once it is realized that ferulate can undergo radical coupling reactions, just like lignin and its monomers can, the next obvious question is, "Can ferulates cross-couple with lignin?" Why is that a significant question? Remember that ferulate is already attached at one end to a polysaccharide. If the ferulate then also becomes attached to lignin, you have cross-linked our nicely degradable polysaccharide to totally undegradable lignin. That is bound to have favorable properties for the plant and is equally certain to have a significant deleterious effect on the plant's degradability.

Now the chemist has an enormous problem. It was easy enough to find ferulates attached to bits of polysaccharides and to be able to release the small diferulates by just cleaving ester bonds and even to show that these diferulates are attached to two chunks of polysaccharides. But now we come to suspect (and, along with other groups, had proof) that these ferulates were attached to lignin. But where are they attached? Why do we care? Well, where they are attached tells you how they got there (the mechanism) and provides insights into controls and alterations that are possible.

Two quite different mechanisms are available for attaching ferulate to lignin, Fig. 7. As it turns out, one of these has been almost universally ignored but doing so has propagated more myths and again results in a tremendous underestimation of this cross-linking role of ferulates.

The first mechanism (we refer to it as the 'passive' mechanism reflecting the relative inactivity of the ferulate in the cross-linking process) has ferulate sitting around in the cell wall watching the lignification occurring around it. At some point the ferulate recognizes that the process of lignification is producing somewhat reactive intermediates with which it could react. In competition with other compounds that might want to react with these intermediates, ferulate may add and form a polysaccharide-ferulate-lignin cross-link, LFP complex A, Fig. 7. This universally accepted mechanism is pleasing in that you can still identify the ferulate in such structures and, by now breaking ester and ether bonds, can release ferulate for quantitation. Ferulate released that way is then a direct measure of cross-linking. Unfortunately, although this is a chemically reasonable mechanism (Scalbert, Monties et al. 1986), it has too many troubling implications (Ralph, Helm et al. 1992; Ralph and Helm 1993). Without going into details, we have sufficient reasons to think that plants would be pretty lame to use such a poor and uncontrolled reaction to effect what is presumably a very important process to the development of the cell wall.

The other mechanism we refer to as 'active' since the ferulate becomes an aggressive player in the lignification reactions. It expands on the notion that ferulate can form radicals — that is how those diferulates above were formed. These radicals could couple with lignin radicals (which are themselves coupling in the normal lignification process) producing a rather complex ferulate-lignin product (LFP Complex B, Fig. 7) from which you can no longer necessarily recognize ferulate as such nor fully release it as anything that can be measured (Ralph and Helm 1993).

So how can you tell what mechanisms are operating and what happens to the ferulate when it becomes attached to lignin? In other words, how on earth can we detect and identify small amounts of ferulate that are now attached to this giant irregular lignin polymer that we have trouble breaking up nicely and characterizing anyway? What we need is some kind of microscope for looking into the molecular world of ferulate.

Fortunately, we have such a molecular microscope. It is called Nuclear Magnetic Resonance Spectroscopy, NMR for short. NMR is an enormously powerful, chemically revealing technique that, unlike other spectroscopies, has a huge variety of different experiments that can yield a dizzying array of information. It does have one significant drawback — NMR suffers from low sensitivity compared with other spectroscopies. We can enhance the sensitivity significantly by judiciously labeling with (non-radioactive) isotopes of carbon (for example). Then we can find out exactly the type of information we are looking for and often do so in a completely unambiguous and incontestable way. Our first step was to determine if ferulates could

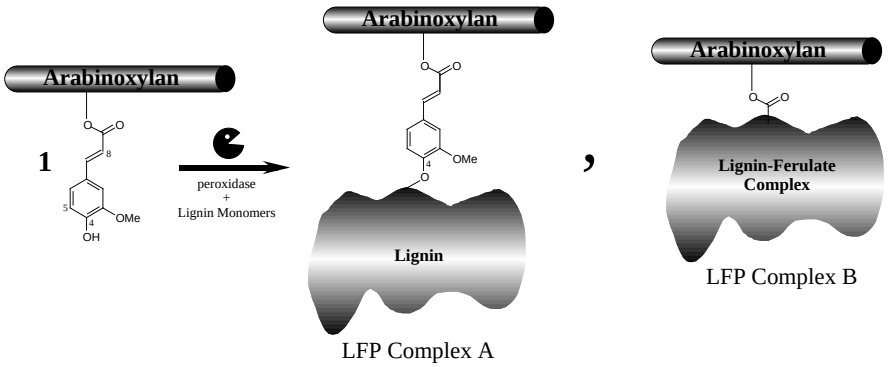

Figure 7. 'Attachment' of ferulates to lignin can proceed by two distinct mechanisms. The 'passive' mechanism (see text) leads to simple α-ethers in a lignin-ferulate-polysaccharide (LFP) complex A. Such ethers are readily quantifiable. The 'active' radical-coupling mechansim incorporates ferulate intimately into the lignin by attachment at 4-O-, 8- and 5-sites producing an LFP Complex B. Of all the possible structures, only β-ethers are then quantifiable.

Understanding this NMR data is actually easy! Indiana Jones would have a 'key' to help him decide if the structures were right.

participate in the lignification process and what kinds of structures would be produced if they did. We did this by making a ferulate model with a truncated polysaccharide to mimic its structure in the plant without all the baggage from the whole big polysaccharide, and allowing that to react with lignin building blocks under normal lignification conditions (Ralph, Helm et al. 1992). Then we used NMR to try to delineate what happened to the ferulate.

The tiny sections from huge two-dimensional spectra shown in Fig. 8a perhaps looks like psychiatrist's ink blot tests; even many chemists don't understand the simplicity and power of the information in them. Look at these as you would a contour map — the more contours the higher the real 3-dimensional peaks and the higher the abundance of that entity. For example, just look at the row of contours labeled as the 8-β products, extracted out into Fig. 8c. What you see here are five pieces of data that all must coincide for the structure you wish to assign to them. Thus, on the vertical axis, the position (chemical shift) of the ester carbonyl carbon (at 177.7 ppm, these values range from about 0 to 210 ppm) must match, and four hydrogen atoms must be at their correct places on the horizontal scale of the spectrum as well as be within three bonds of this carbonyl carbon in the molecule. Wow! Five concurrent pieces of data that are required to match. Indiana Jones would have a metal key that would fit exactly over the required data. This is illustrated at the bottom of Figure 8; The key would really be much much bigger since this is only a tiny part of the spectrum. The peaks must fall exactly in the slots in the key to be a match. Needless to say, finding another chemical structure that would have all of these coincident data is highly improbable. That is why we can be secure in knowing that assignments made by these methods are essentially unambiguous. You can see the data from a model compound, a small synthetic molecule that has all the same structural features as the one in the polymer, are sharper but exactly match the polymer data, Fig. 8b.

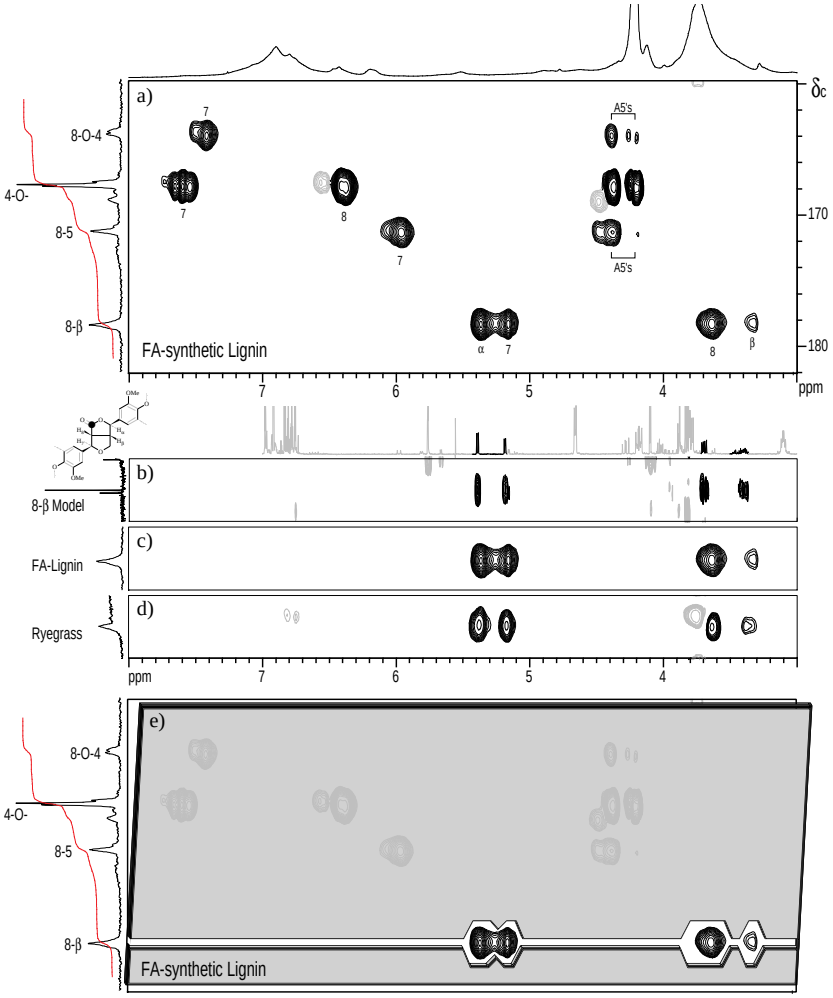

Figure 8. NMR spectra of ferulates in lignins of various types. The non-important peaks (for this discussion) are greyed out. a) Ferulate in a synthetic lignin showing all of the types of coupling products that ferulate can end up in. b) Section showing correlations in an 8-β model compound. c) just the same 8-β region of the synthetic lignin in a. d) The same 8-β region in ryegrass lignin, showing that these structures are clearly present in real lignin. e) tries to illustrate the "Indiana Jones" Key idea. In order to be the structure we expect (and have models for) the signals must all fit in the gaps in the key.

Now that we know what kinds of structures are produced and have their NMR data, we can look for these structures in real plant materials. That would provide evidence for our 'active' mechanism. This is where we run into our NMR insensitivity problem. However, by growing plants in an atmosphere in which the carbon dioxide is enriched to about 15% in $^{13}CO_2$ (natural abundance is 1%), we gain a 15-fold NMR sensitivity increase that makes the detection of these structures simple (Ralph, Grabber et al. 1995). Thus, Fig. 8d shows that the 8-β structure is obviously present in ryegrass. This structure can only be formed by the 'active' mechanism.

So, the NMR experiments show that our 'active' mechanism is indeed used by plants. This means that the plant is

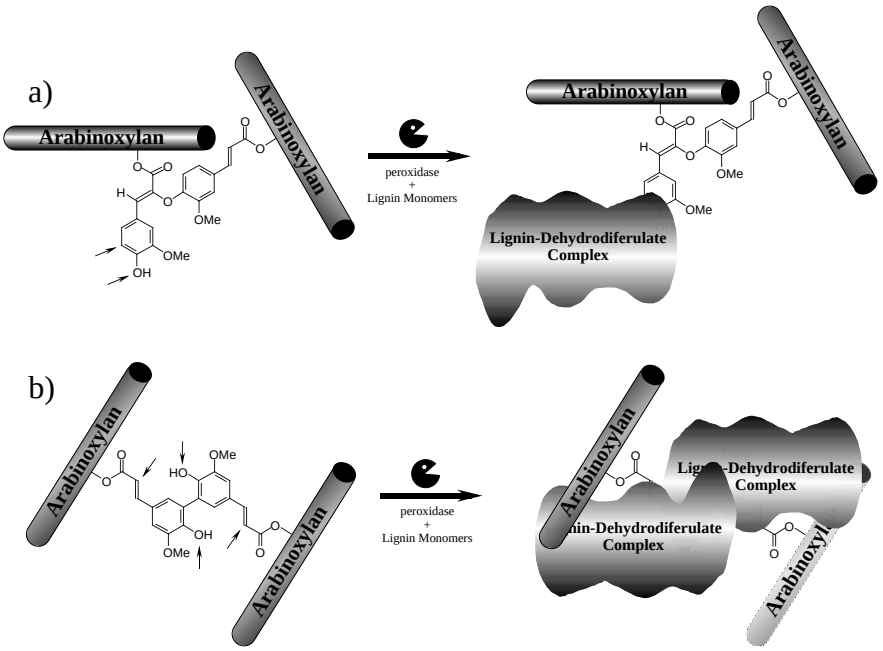

Figure 9. Ferulate dimers, already cross-linking polysaccharide (arabinoxylan) chains, can also incorporate into lignins via active mechanisms. Schemcatically shown are incorporation of a) the 8-O-4 diferulate and b) the 5-5 diferulate which can produce a very highly cross-linked matrix.

controlling these crucial cross-linking mechanisms to give the greatest structural integrity. It also means, however, that the poor scientists who thought they were measuring cross-linking by measuring etherified ferulates were missing a big part of the cross-linking picture since only one of the possible cross-linking structures will actually release ferulate. Recent evidence suggests that ferulates involved in cross-linking are about 250% of what is measured as etherified ferulates (Grabber, Hatfield et al. 1995). Again, the role of ferulate in the growth, development, and architecture of the cell wall is substantially more important than previously recognized.

Two more things. Just to make things even more complex (but logical from the plant's point of view), diferulates, which already have tied two polysaccharide chains together, can also become involved in the lignification reactions and consequently produce a very highly cross-coupled lignin polysaccharide network, Fig. 9 (Ralph, Hatfield et al. 1996; Ralph, Hatfield et al. 1996). It is not surprising that these chemical associations of lignins with polysaccharides render the polysaccharide considerably less available for digestion. In a basic sense, an even more striking fact was revealed by the NMR studies of the ryegrass lignin (Ralph, Grabber et al. 1995). Ferulate doesn't just participate in lignification — it is the site at which lignification starts! It is a nucleation site from which the lignin polymer grows from its anchor on the polysaccharide. Thus, the plant is utilizing ferulate as a way of directing lignification to the appropriate sites in the cell wall and orchestrating the initial process.

Although we don't understand all the details of ferulate regulation and placement, the plant can obviously control ferulate attachment to its polysaccharides. Clearly the plant then has control over how the lignification process is directed to specific sites in the wall and over the cross-linking of its polysaccharides to lignin.

Digestibility Implications

What do all these revelations about ferulate mean to digestibility? First, just dimerizing ferulates already has a significant impact on both the rate and extent of polysaccharide degradability, Fig. 10_{left} (Grabber, Hatfield et al. 1996; Grabber, Ralph et al. 1996; Hatfield, Grabber et al. 1996). Cross-coupling the ferulate to lignin during lignification is even more dramatic, Fig. 10_{right}, and produces a formidable challenge to our poor cow. It is also clear that ferulate has an enormously significant role in the growth and development of grass walls. It is therefore a logical choice for manipulation via standard breeding or genetic manipulation methods. Indeed, it would appear to us that this little molecule, at the heart of reactions which tie polysaccharides to lignin, is of far greater con-

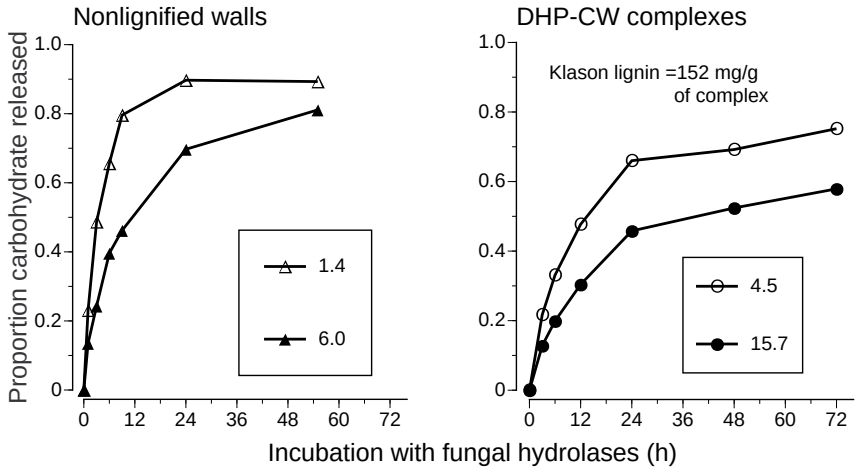

Figure 10. Carbohydrates released from non-lignified and lignified cell wall samples with different diferulate and ferulate levels. Left: varying diferulate contents, 1.4 vs. 6.0 mg/g of CW, the total ferulate levels were similar. Right: varying ferulate levels after the same lignification, 4.5 mg/g of ferulate vs. 15.7 mg/g. Polysaccharide cross-linking via ferulate dimerization alone (left) depresses the rate and possibly extent of polysaccharide degradation. After lignification (right), each is depressed further, the sample having the highest ferulate (highest cross-linking) levels (15.7 mg/g) has significantly depressed degradability.

Ferulates provide the very sites at which lignification starts.

"...ferulate is so mechanistically critical to the development of the wall that there may be limitations to the amount of down-regulation that can be tolerated."

"Understanding biochemical mechanisms provides a sound basis for the application of standard breeding selections or genetic biotechnologies to the improvement of plants for targeted uses."

Acknowledgement

Many thanks to artistic nephew Andy Muenchow for the cartoon on page 3, which is significantly better than my feeble attempt!

sequence to degradability than the nature of lignin itself (which is the focus of most of the transgenic studies worldwide). Hans Jung will address more of these issues in the following article. Also clear, however, is that although it may be possible to mess with lignin monomers and not affect the viability and integrity of the plant system, ferulate is so mechanistically critical to the development of the wall that there may be limitations to the amount of down-regulation that can be tolerated. Since grasses utilize ferulate to direct lignification as well as effect extensive cell wall cross-linking, the plant may not take kindly to losing this critical element. Or is the plant resilient enough to find alternative ways to effect similar changes? As far as we know, woody plants do not use ferulates; no similar mechanisms have been uncovered. Or have researchers missed the critical elements that provide woody plants with similar schemes to those we have uncovered in grasses? Obviously they successfully create cell walls with the appropriate properties. These studies even point to the possibility of engineering lignin to be more easily degraded chemically and may eventually lead to lower input pulping.

Conclusions

Understanding mechanisms is ultimately superior to empirical correlative observations. Mechanisms allow one to not only rationalize current observations but also to predict new scenarios. Understanding biochemical mechanisms provides a sound basis for the application of standard breeding selections or genetic biotechnologies to the improvement of plants for targeted uses. Current approaches to lignin modification may prove fruitful and should therefore be encouraged. However, the above considerations suggest that approaches regarding ferulate will have a greater effect as ferulate is a more crucial element in controlling wall development that is likely to impact digestibility. In addition to enhancing our understanding of the limitations to digestibility, it is hoped that these fundamental studies will provide avenues for the production of cell walls with end-use-targeted properties.

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